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Principal parameters affecting virus inactivation by the solar photo-Fenton process at neutral pH and μM concentrations of H_2O_2 and $Fe^{2+/3+}$

E. Ortega-Gómez [a,b], M.M. Ballesteros Martín [b,d], A. Carratalà [c], P. Fernández Ibañez [b,e], J.A. Sánchez Pérez [a,b], C. Pulgarín [f,*]

[a] Department of Chemical Engineering, University of Almería, 04120 Almería, Spain
[b] CIESOL, Joint Centre of the University of Almería-CIEMAT, 04120 Almería, Spain
[c] Laboratory of Environmental Chemistry, School of Architecture, Civil and Environmental Engineering (ENAC), École Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland
[d] Department of Molecular Biology and Biochemical Engineering, University of Pablo de Olavide, 41013 Sevilla, Spain
[e] Plataforma Solar de Almería, CIEMAT, 04200 Tabernas, Almería, Spain
[f] École Polytechnique Fédérale de Lausanne, Institute of Chemical Sciences and Engineering GPAO, Station 6, CH-1015 Lausanne, Switzerland

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ABSTRACT

The inactivation of the coliphage MS2 (a human virus indicator) by solar photo-Fenton at near neutral pH in carbonate buffer solution matrix was evaluated. The effects of reactant concentration (H_2O_2, Fe^{2+}, Fe^{3+}) and solar irradiance on the photo-Fenton process were studied. Specifically, the solar exposure/Fe^{3+} treatment showed a strong dependence on the iron concentration and solar irradiance intensity leading to complete inactivation (from 10^6 PFU mL^{-1} to the detection limit) with 1 mg L^{-1} of Fe^{3+} and 60 min of solar irradiance (45 W m^{-2}). The MS2 inactivation observed with the photo-Fenton process (solar exposure/H_2O_2/$Fe^{2+/3+}$) carried out with Fe^{3+}, was faster than with Fe^{2+} (detection limit achieved at 20 min and 50 min, respectively). Moreover, virus inactivation by photo-Fenton under different solar irradiance values (15, 30 and 45 W m^{-2}), H_2O_2 and Fe^{3+} concentrations (0.1, 0.5 and 1 mg L^{-1}) and different pH values (6, 7 and 8) were evaluated. In order to validate the efficiency of the photo-Fenton process at near neutral pH on virus inactivation, the photocatalytic treatment was carried out in natural water (Lake Geneva, Switzerland) and with a human virus (Echovirus). Finally, a conceptual mechanistic interpretation was proposed regarding how solar photo-Fenton acts on viruses in water, involving the key species Fe^{2+}, Fe^{3+}, H_2O_2, solar irradiance, organic matter, and their possible reactions and interactions.

1. Introduction

Advanced oxidation processes (AOPs) have been put forward in recent years as physicochemical alternative treatments based on the generation of strongly oxidizing radicals that are very effective in organic matter oxidation. Amongst them, the photo-Fenton process is of special interest since it has been applied to the treatment of a large variety of hazardous contaminants as well as the inactivation of a wide range of harmful microorganisms in water and it is able to use solar radiation as an energy source [1,2]. During the Fenton reaction, hydrogen peroxide rapidly reacts with iron, and generates hydroxyl radicals, which are a non-selective and highly oxidative species. Iron is added at the beginning of the process and acts as a catalyst, being both oxidized and reduced continuously. The presence of the photons leads to the generation of more hydroxyl radicals and regenerates Fe^{2+} [3]. The optimal pH of the photo-Fenton process is 2.8, although the operational costs associated with acidification of the large volumes of wastewater to be treated are very high and the need for subsequent neutralization increases the salinity of treated water. However, recent research has demonstrated the possibility of carrying out the photo-Fenton reaction at neutral or near neutral pH [4] reducing its operational costs and making this process simpler to use. The solar photo-Fenton process at near neutral pH has been recently investigated and it has proved to be a promising treatment for the disinfection of water containing bacteria, fungi, etc. [5–10]. However, virus inactivation using photo-Fenton has hardly been investigated until now [11–13].

* Corresponding author. Tel.: +41 21 693 47 20.
E-mail address: cesar.pulgarin@epfl.ch (C. Pulgarín).

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Phages are more similar to human virus than other commonly used bacterial indicators [14]. These organisms have many essential properties in common with human enteric viruses in terms of composition, morphology, structure, size or site replication and they are also present in contaminated fecal water [14,15].

According to the World Health Organization (WHO) water quality guidelines [16], bacteriophages (phages), which are bacterial viruses ubiquitous in the environment, are recognized and recommended as a human enteric virus model and for water quality testing. The use of phages as models for pathogenic enteric microorganisms has been recognized since the 1930 s and a direct correlation between the presence of certain bacteriophages and the intensity of fecal contamination has been reported elsewhere. As such, phages are a reliable tool for research on viral inactivation since they don't pose any human health risks, as occurs with human viruses such as *Echovirus*, which is associated with gastroenteritis, encephalitis and meningitis [17,18].

Bacteriophage MS2 is a single-stranded RNA, frequently used as model for enteric viruses due to the similarities they have regarding their size and structure [19]. The MS2 virus is formed by a protein shell that serves to protect the ss-RNA viral genome. MS2 only infects *Escherichia coli* bacteria through A protein which allows adhesion to the bacteria pili and then the virus RNA is injected into the bacteria [20]. MS2 is a very resistant phage because its small size prevents virus removal through physical treatments such as sedimentation, coagulation and conventional filtration [21,22]. In addition to this, viruses are generally more resistant than other pathogens to many disinfectants, such as chlorine [23]. In the last few years, some researchers have investigated the MS2 inactivation by photo-Fenton although complete virus removal was not achieved [11–13].

The aim of this research was to study virus inactivation by the photo-Fenton process at near neutral pH in water. With this purpose in mind, individual effects of solar irradiance, concentrations of H_2O_2 and $Fe^{2+/3+}$ and pH on virus inactivation by photo-Fenton were studied. All the experiments were conducted with MS2 coliphage as a human virus indicator. To confirm the efficiency of photo-Fenton process at neutral pH in real conditions, inactivation of MS2 coliphage in natural lake water from Lake Geneva (Switzerland) was assayed. Additionally, the inactivation of an human virus (*Echovirus*) in carbonate buffer solution was also studied. Based on the experimental results and a bibliographical revision, a mechanistic interpretation is proposed which illustrates the possible pathways involved in MS2 virus inactivation by photo-Fenton treatment.

2. Material and methods

2.1. Reagents

Hydrogen peroxide (H_2O_2, 30% w/w), iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), yeast extract, D-glucose (99%) and streptomycin sulfate were purchased from Sigma–Aldrich. Sodium chloride (NaCl, 99%), calcium chloride ($CaCl_2$, 99%), and chloroform (99.8%) were acquired from Acrõs Organics. Iron (III) chloride ($FeCl_3$, 98%), sodium bicarbonate ($NaHCO_3$, 99%) and bactotryptone were supplied by Abcr, Fluka and Becton Dickinson, respectively.

2.2. Virus strain and growth media

MS2 coliphage (DSMZ 13767) and its bacterial host, *E. coli* (DSMZ 5695), were acquired from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). The propagation of MS2 coliphage was performed in 1 L of LB medium (10 g of bactotryptone, 1 g of yeast extract, 8 g of NaCl, 1 g of D-glucose, 0.3 g of $CaCl_2$ and 2 mg of streptomycin sulfate) containing *E. coli*. When the optical density of this culture reached 0.4 measured at 600 nm, *E. coli* was inoculated with the MS2 virus at a multiplicity of infection (ratio between phage and bacteria) of 0.1. After five hours of incubation at 37 °C, the bacterial cells were lysed with chloroform (5 mL) and immediately purified. The concentration of infective MS2 was determined by a double-layer agar method and expressed as plaque forming units per mL (PFU mL^{-1}). The MS2 stock concentration was 10^9 PFU mL^{-1} and the initial concentration used in the reactor was 10^6 PFU mL^{-1}.

Human virus, *Echovirus* 11 stocks were produced by infecting BGMK cells cultured in minimum essential medium (MEM) supplemented with 1% glutamine, 50 μg of gentamycin per ml and 10% (growth medium) or 2% (maintenance medium) of heat-inactivated fetal bovine serum (FBS). Viruses were released from cells by freezing and thawing the cultures three times. After that, a centrifugation step at 3.000× *g* for 20 min was applied to eliminate cell debris. Viruses were ultra-centrifuged at 34.500× *g* and the obtained pellet was re-suspended in PBS without further purification. Finally, viral suspensions were quantified and stored in 10 ml aliquots at −20 °C until use. In all the assays, the concentration of infective *Echovirus* was determined by the most probable number method (MPN) and the results were expressed as most probable number cytophatic units per mL, MPNCU mL^{-1} [24]. The *Echovirus* stock concentration was 10^7 MPNCU mL^{-1} and the initial concentration used in the reactor was 10^4 MPNCU mL^{-1}.

2.3. Water matrices

Two different types of water matrices were used for the inactivation experiments: i) carbonate-buffered saline (CBS, 0.1 mM $NaHCO_3$ + 15 mM NaCl) at pH 8 and ii) Natural water collected from Lake Geneva (Switzerland). Prior to use, the natural water was filtered under vacuum conditions using 0.45 μm filters (Millex®, MILLIPORE) and later sterilized in order to eliminate the solids in suspension and remove other microorganisms. The pH of the lake water was close to 8.5.

2.4. Experimental setup

All the experiments were performed in glass reactors containing 100 mL of the water matrix. The initial concentration of infective MS2 was 10^6 PFU mL^{-1}. The solution was continuously stirred with a magnetic stir bar at 350 rpm. For the experiments in dark conditions, the reactors were covered with aluminum foil. The experiments under sunlight were carried out using a solar simulator (SUNTEST CPS, Heraeus, Germany) with IR and UV-C cut-off filters to simulate solar global radiation from outdoor daylight and avoiding the influence of UV-C radiation and thermal heating. The solar UVA radiation intensities employed in the assays were 15, 30 and 45 W m^{-2}. These values represent typical intensities achieved by UVA solar light for different seasons. An irradiance of 30 W m^{-2} was selected in the experiments carried out with a constant solar UVA irradiance in order to extrapolate the results to real conditions, since this irradiance is considered a media value of global UV irradiance under clear skies in sunny countries [1,25]. The temperature was constant throughout the experiments (33 ± 1 °C). All the material and solutions were sterilized at 126 °C for 20 min.

2.5. Inactivation experiments

At the beginning of each experiment, the reactors containing the water matrix and virus suspension were placed in the dark and stirred for 5 min. The reactors were then spiked with 0.1, 0.5 or 1 mg L^{-1} of $Fe^{2+/3+}$ or/and 0.1, 0.5 or 1 mg L^{-1} of H_2O_2 from freshly prepared stock solutions to achieve the desired concentration

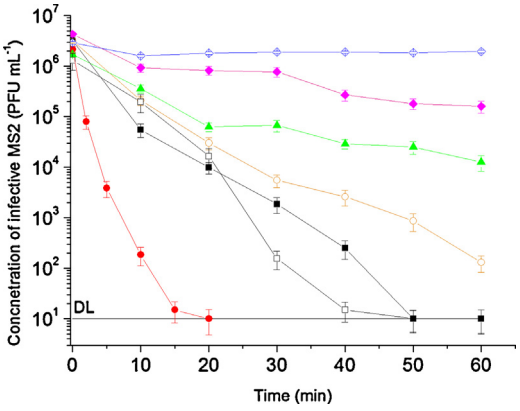

Fig. 1. MS2 Inactivation in CBS at pH 8 by the combination of H_2O_2/Fe^{2+} (Fenton reaction) (-◆-), H_2O_2/Fe^{3+} (-▲-), solar exposure/H_2O_2 (-□-), solar exposure/Fe^{3+} (-◌-), solar exposure/H_2O_2/Fe^{2+} (photo-Fenton reaction) (-■-) and solar exposure/H_2O_2/Fe^{3+} (photo-Fenton reaction) (-●-). Average of H_2O_2, Fe^{2+}, Fe^{3+} and UVA radiation individual effect on MS2 viability (-◇-). (H_2O_2 = 1 mg L^{-1}, $Fe^{2+/3+}$ = 1 mg L^{-1} and I_{UV} = 30 W m^{-2}).

according to the experimental conditions. Finally, for the photo-inactivation experiments the lamp was turned on. The samples were exposed to the desired conditions for 60 min. During the experiments, samples were taken at predetermined times and immediately diluted in CBS. All experiments were conducted in triplicate, so that the confidence level of the results was higher than 95% (ANOVA analysis). In all water samples that reached the detection limit, the viral regrowth was assessed by plating 100 μL of each aliquot of the last water sample in the adequate culture media 24 h after the photo-inactivation experiment was ended and any viral regrowth was observed after 24 h.

3. Results and discussion

3.1. Influence of H_2O_2, Fe^{2+}, Fe^{3+}, solar exposure and their combinations on MS2 inactivation

3.1.1. Effect of the individual parameters: H_2O_2, Fe^{2+}, $Fe^{3+}$and solar irradiance

The water matrix (sterile carbonate-buffer solution, CBS) in the dark and without any reagent had no effect on MS2 viability. Under the same conditions, but adding 1 mg L^{-1} of H_2O_2, no virus inactivation was detected by the action of the oxidant agent (Fig. 1). These results are in concordance with others found in the literature where no inactivation of MS2 was obtained with a higher H_2O_2 concentration (17 mg L^{-1}) at the same initial pH and over the same experimental time [26] or with a similar initial concentration of H_2O_2 (0.9 mg L^{-1}) [11]. The disinfectant capacity of the H_2O_2 is well known, e.g., the hydrogen peroxide vapor (HPV) can be used against enteric and respiratory viruses [27]. However, low H_2O_2 concentrations, as used in this study did not cause any virucidal effect in the MS2 coliphage.

Similarly, the exposure to 1 mg L^{-1} of Fe^{2+} or Fe^{3+} in the dark didn't give rise to any kind of detrimental effect on virus viability over a 60 min period (Fig. 1). An earlier study showed a 1.5-log inactivation using a higher initial concentration of Fe^{2+} (5.5 mg L^{-1}) while non-inactivation took place using the same concentration of Fe^{3+} in the dark [28]. When Fe^{2+} is present in water at pH 8, the hydrolyzed ferrous iron species $[Fe(OH)_2(H_2O)_4]$ is mainly present in solution [29]. On the contrary, at this pH, Fe^{3+} is mostly in the form of iron(oxy) hydroxide as described elsewhere [11,12,30]. These authors demonstrated that there is an adsorption of the ferric oxide particles onto the viral surface. The virus-particle aggregation is probably a consequence of hydrophobic interactions or Van der Walls forces [31] and electrostatic interactions, since the Fe^{3+} can serve as an electron acceptor and the MS2 coliphage is negatively charged at pH 8 [30]. Some authors have demonstrated that the presence of metal oxides favors the physical adsorption of the virus, although this effect doesn't lead to any inactivation, as confirmed by our results [11,30].

Exposure of MS2 suspensions to an irradiance of 30 W m^{-2} for 60 min did not cause any inactivation (Fig. 1), which is in line with previous findings on MS2 as a virus highly resistant to solar radiation [32]. A number of studies have shown that MS2 inactivation is brought about by UV exposure, although most of them were conducted with UV-C lamps, whose spectral range is not present in solar radiation reaching the Earth surface [33–35]. As far as we know, our results can only be compared with the research of [32], as this study was done with wavelengths >295 nm, belonging to the UVA part of solar radiation. These authors demonstrated that the viruses T4 and T7 can be severely inactivated by the mere action of solar UVA radiation. This inactivation is caused by viral genome (inhibiting both RNA replication inside the host cell and genome injection) and protein damage leading to a loss of infectivity [32]. The inhibition can be attributed to an absorption of photons by virus components, that lead to chemical modification of the genetic material, e.g. dimer formation in RNA structure and other photoproducts that *E. coli* cells hosts cannot repair [36,37] or damage to proteins resulting in photo-oxidation of select amino acid residues [38]. It was demonstrated by Davies-Colley *et al.* [39] that UVB (290–320 nm), UVA (320–400 nm) and blue to green visible light (400–550 nm) contribute to f-RNA phage damage. However, some of the mechanisms that restrain the infection capacity can be repaired. The repair mechanisms are especially significant in the MS2 coliphage since it is one of the most resistant viruses (excluding adenovirus) to UV inactivation [40]. Furthermore, wavelengths between 370 and 550 nm are responsible for restoring infectivity by inducing light repair mechanisms in some viruses through removal of pyrimidine dimers [41]. In general, factors that may be involved in virus resistance to UV irradiation are high guanine and cytosine content [42].

3.1.2. Effect of H_2O_2/Fe^{2+} (Fenton treatment) and H_2O_2/Fe^{3+}

Slight differences in concentration of infective MS2 coliphage were observed by combining Fe^{2+}/Fe^{3+} and H_2O_2 (Fig. 1). Fenton treatment carried out with 1 mg L^{-1} of Fe^{2+} and 1 mg L^{-1} of H_2O_2 under dark conditions produced only a 1.5-log decrease in the concentration of infective MS2 within 1 h. However, when Fe^{3+} was the source of iron, an improved inactivation of 2.1-log was attained. According to the Fenton reaction, where the main species responsible for the oxidation are hydroxyl radicals ($HO^{\bullet}$), the virus inactivation rate should be higher in the presence of Fe^{2+} (Eq. (1)) than with Fe^{3+} (Eq. (2)) [43]. However, in the last few years, some researchers have suggested that an alternative oxidant, such as ferryl ion (Fe^{4+}), could be formed under neutral pH conditions during the Fenton reaction [44]. At neutral pH, Fe^{2+} is mainly present as an inorganic complex (Eqs. (3)–(6)) although the identity of the oxidant produced in the subsequent steps is still uncertain. As such, there are controversial contributions to the literature regarding the role of the main oxidative reactive intermediates in the Fenton reaction at neutral pH [44–49].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^- \quad k = 63 - 73\ M^{-1}s^{-1} \qquad (1)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\bullet} + H^+ \quad k = 0.001 - 0.01\ M^{-1}s^{-1} \qquad (2)$$

$$Fe^{2+} + 6H_2O \rightarrow [Fe(H_2O)_6]^{2+} \qquad (3)$$

$$[Fe(H_2O)_6]^{2+} + HO^- \rightarrow [Fe(OH)(H_2O)_5]^+ + H_2O \qquad (4)$$

$$[Fe(OH)(H_2O)_5]^+ + HO^- \rightarrow [Fe(OH)_2(H_2O)_4]_{(aq)} + H_2O \qquad (5)$$

$$[Fe(OH)(H_2O)_5]^+ + H_2O_2 \rightarrow [Fe(OH)(H_2O_2)(H_2O)_4]^+ + H_2O \quad (6)$$

$$[Fe(OH)(H_2O_2)(H_2O)_4]^+ \rightarrow [Fe(OH)(H_2O)_4]^{2+} + HO^\bullet + OH^- \quad (7)$$

$$[Fe(OH)(H_2O_2)(H_2O)_4]^+ \rightarrow [Fe(OH)(H_2O)_4]^{3+} + 2OH^- \quad (8)$$

Some authors suggest that the iron complex may dissociate to form HO• (Eq. (7)) or a ferryl species (Eq. (8)) which can also form HO• in water [44]. Moreover, the conditions of this aqueous solution, like pH and the presence of ligands, are crucial in Eqs. (7)–(8). This could clarify the differences in viral inactivation rate between Fe^{2+} and Fe^{3+} during the Fenton reaction, as other authors observed for bacteria [4] and spores [8]. Regardless of the oxidative species at work during Fenton process, their generation very close to the viral particle is more likely to cause damage. Even, Nieto-Juárez *et al.*, [11] suggested that the virus is only susceptible to inactivation when Fe^{3+} is adsorbed onto the viral particle.

3.1.3. *Effect of solar irradiance/H_2O_2*

The combination of 30 W m^{-2} of solar UVA irradiance with 1 mg L^{-1} of H_2O_2 leads to total virus inactivation after 50 min of solar exposure (Fig. 1); whereas each treatment used in isolation did not cause any significant inactivation for the same time period. These results provide evidence for the action of a synergetic effect between solar exposure (>400 nm) and H_2O_2, as previously found for bacteria and fungi. In natural urban wastewater using solar radiation and a higher H_2O_2 concentration (20 mg L^{-1}), somatic coliphages and F-specific RNA bacteriophages showed a reduction of 2-log and 2.5-log, respectively [13]. This effect is different to that produced when short wavelength photons (200–280 nm) lead to homolytic rupture of H_2O_2 to generate hydroxyl radicals [33,34]. This spectral range is not present in solar radiation, therefore it cannot be the mechanism acting when solar/H_2O_2 treatment is used. Under solar irradiation, modifications produced in the genetic material can be repaired in the host cell. At the same time, the presence of a strong oxidant agent, such as hydrogen peroxide, could cause modifications or damage to the virus surface structure. These modifications or damage mainly affect the protein coat (A protein) or the attachments sites needed for infection of *E. coli* host cells [50]. This leads to interferences in the reparation mechanisms. Therefore, this synergistic effect could be explained by the damage caused as a result of the solar irradiation that makes the virus more sensitive to the action of the oxidant agent instead of hydroxyl radical action.

3.1.4. *Effect of solar exposure/$Fe^{2+/3+}$*

The action of 1 mg L^{-1} of Fe^{3+} under solar radiation (30 W m^{-2}) induced to a 3.5-log decrease in virus concentration, whereas when the same amount of Fe^{2+} and irradiance were assayed, any significant loss of coliphage infectivity was detected (Fig. 1). These results confirm the fundamental role of the source of iron during photo-inactivation of the MS2 coliphage [11,12] and were also corroborated for fungi (*Phytophthora capsici*) by [8]. Some studies have demonstrated that exposure of virus to UV in the presence of iron particles adsorbed onto its surface leads to coliphage inactivation [12,30,51]. However, an exhaustive study of the main parameters affecting solar/Fe^{3+} process for MS2 virus inactivation has not been done up to now. With this in mind, for this study, different solar UVA radiation intensities (15, 30 and 45 W m^{-2}) were evaluated using the same Fe^{3+} concentration (1 mg L^{-1}). As Fig. 2a shows, after 60 min of experiment, the concentration of infective MS2 decreased 1 and 4-log at 15 and 30 W m^{-2}, respectively, whilst for 45 W m^{-2} of solar UVA irradiation the detection limit was reached within the same time. Moreover, three initial concentrations of Fe^{3+} (0.1, 0.5 and 1 mg L^{-1}) were evaluated using the same solar irradiation intensity (30 W m^{-2}) with the aim of studying the iron concentration effect on the solar/Fe^{3+} process. In this case, significant

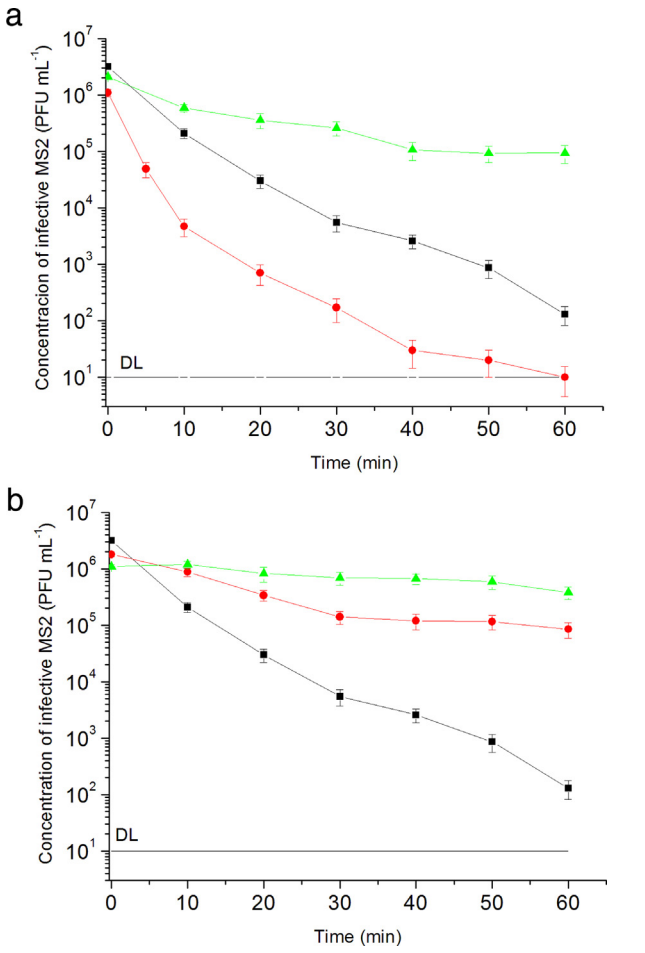

Fig. 2. MS2 Inactivation at pH 8 with: (a) 1 mg L^{-1} of Fe^{3+} and 15 (–▲–), 30 (-■-) and 45 (–●–) W m^{-2} of solar irradiance intensity; (b) 30 W m^{-2} of solar irradiance intensity and 0.1 (–▲–), 0.5 (–●–) and 1 (-■-) mg L^{-1} of Fe^{3+}.

differences were also observed (Fig. 2b), 0.5, 1.1 and 4.2-log MS2 infectivity decreases were observed respectively, when 0.1, 0.5 and 1 mg L^{-1} of Fe^{3+} were added.

Due to the structure and composition of the viruses, the iron-virus aggregate can act as a sensitizer favoring light absorption and resulting in a ligand-to-metal charge-transfer (LMCT) which leads to the production of Fe^{2+} and oxidized ligands that react with the O_2 generating ROS. These ROS, superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), peroxyl ($RO^\bullet_2$), alcoxyl ($RO^\bullet$) and hydroperoxyl, cause damage to the viral constituents [52]. Another possible pathway could be the semiconductor role of iron (hydr)oxide. The iron (hydr)oxide could act as a semiconductor with a reduce band gap and may be photo active under solar irradiation. Electrons and holes that are photo generated in the iron oxide semiconductor lattice are scavenged by surface sites to produce HO• radicals and Fe^{2+} [53]. These mechanisms give rise to an indirect endogenous damage in the virus. This occurs because photons are absorbed by one part of the virus, but damage is conveyed to another region of the virus and consequently ROS are produced [38]. However, the synergetic effect was negligible in the presence of Fe^{2+} and solar irradiance, since the formation of photo-active complex was not favored and any loss of viability was observed in the coliphage MS2.

As the results show, when the solar irradiance increases, at the same iron concentration, the generation of oxidant species is favored and consequently higher virus inactivation levels are achieved (Fig. 2a). Afterwards, for different Fe^{3+} concentrations

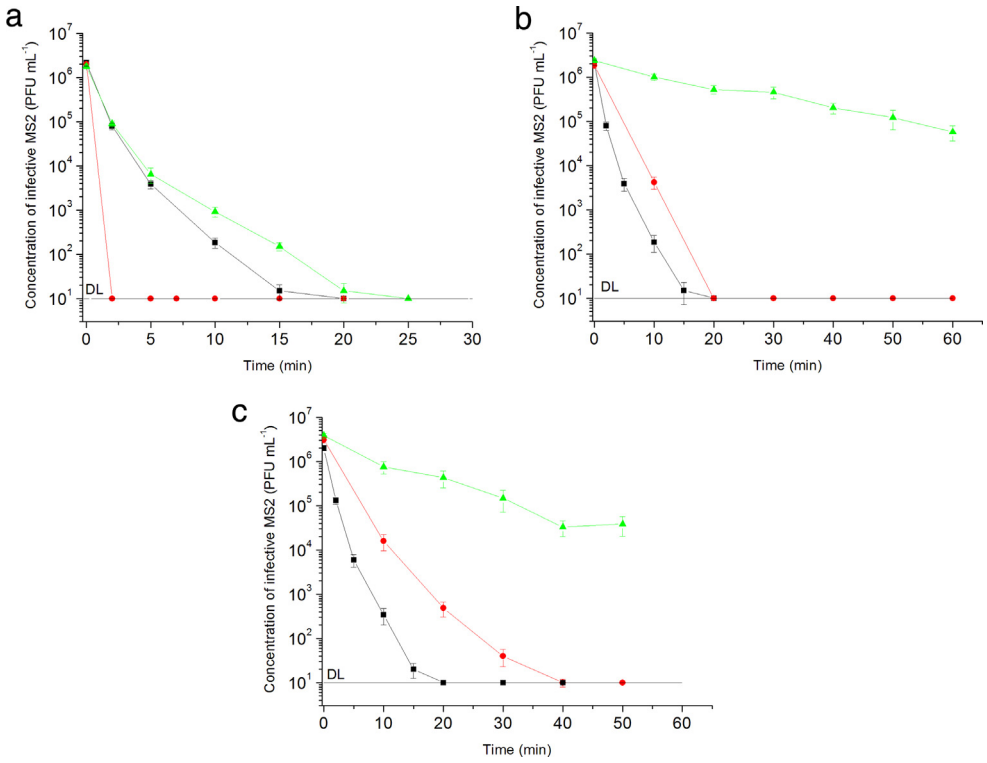

Fig. 3. MS2 Inactivation by photo-Fenton process at pH 8 with: (a) 1 mg L^{-1} of H_2O_2, 1 mg L^{-1} of Fe^{3+} and under 15 (- ▲ -), 30 (-■-) and 45 (- ● -) W m^{-2} of solar intensity; (b) 1 mg L^{-1} of Fe^{3+}, under 30 W m^{-2} of solar intensity and 0.1 (- ▲ -), 0.5 (- ● -) and 1 (-■-) mg L^{-1} of H_2O_2; (c) 1 mg L^{-1} of H_2O_2, under 30 W m^{-2} of solar intensity and 0.1 (- ▲ -), 0.5 (- ● -) and 1 (-■-) mg L^{-1} of Fe^{3+}.

under the same solar irradiance (Fig. 2b), the availability of iron was limited at low concentrations disfavoring the production of ROS which increase the concentration of infective coliphage MS2 (Fig. 2b). These results suggest that both irradiation intensity and iron concentration are critical in this process. If more photons and iron are available in the system, better inactivation results are achieved, probably due to a higher generation rate of oxidative species responsible for the MS2 virus inactivation.

3.2. MS2 inactivation by the photo-Fenton process

During photo-Fenton (1 mg L^{-1} of Fe and 1 mg L^{-1} of H_2O_2 under UVA exposure of 30 W m^{-2}) the differences between using ferrous or ferric ions were noticeable (Fig. 1). Using Fe^{3+} the detection limit was reached in 20 min, however, 50 min were needed to achieve complete inactivation when the source of iron was Fe^{2+} at the same concentration. The different behavior between the photo-Fenton carried out with Fe^{2+} or Fe^{3+} can be explained as follows.

When Fe^{3+} was used, solid iron species such as (hydr)oxide in colloidal form are photo-catalytically converted to ferrous ions (Fe^{2+}) via LMCT, along with the formation of an additional equivalent of the hydroxyl radical (Eq. (12)) in the presence of solar irradiation. The Fe^{2+} formed in Eq. (12) reacts with the H_2O_2 and is regenerated by the photo-Fenton process. Apart from that, intermediate complexes dissociate are formed, as shown in Eq. (13). Depending on the reacting ligand, the product may be a hydroxyl radical, such as in Eqs. (14)–(15), or another radical derived from the ligand Eq. (16) [54]. In the presence of H_2O_2 heterogeneous photo-Fenton may take place when the oxidant species are generated close to the virus. These are more likely to damage the virus structure when the virus-iron particles are in aggregated form. In the photo-Fenton with Fe^{2+}, the possible oxidant species formed by the Eqs. (1), (9)–(16) are probably generated further away from the viruses. However at neutral pH, the amount of Fe^{2+} in solution is negligible unless the Fe^{2+} is complexed with organic matter or with possible metabolites derived from the degradation of viral constituents [55]. These results are in agreement with previous works where commercial iron (hydr)oxide particles which contain particles of Fe^{3+}, caused more rapid virus inactivation with respect to particles containing Fe^{2+} during photo-Fenton treatment [11]. Polo-López *et al.*, [8] also found similar differences between the use of Fe^{3+} and Fe^{2+} during the solar inactivation of *P. capsici* in water at low iron concentrations from 1 to 5 mg L^{-1}.

$$Fe^{2+} + HO^{\bullet} \rightarrow Fe^{3+} + HO^{-} \quad (9)$$

$$3HO^{\bullet} + RH \rightarrow H_2O + R^{\bullet} \quad (10)$$

$$R^{\bullet} + Fe^{3+} \rightarrow R^{+} + Fe^{2+} \quad (11)$$

$$Fe^{3+} + H_2O + h\nu \rightarrow Fe^{2+} + H^{+} + HO^{\bullet} \quad (12)$$

$$[Fe^{3+}L] + h\nu \rightarrow Fe^{2+} + L^{\bullet} \quad (13)$$

$$[Fe(H_2O)]^{3+} + h\nu \rightarrow Fe^{2+} + HO^{\bullet} + H^{+} \quad (14)$$

$$[Fe(OH)]^{2+} + h\nu \rightarrow Fe^{2+} + HO^{\bullet} \quad (15)$$

$$[Fe(OOC\text{-}R)]^{2+} + h\nu \rightarrow Fe^{2+} + CO_2 + R^{\bullet} \quad (16)$$

The most effective process (solar irradiation/H_2O_2/Fe^{3+}) was studied in detail in order to understand the mechanisms involved in the loss of infectivity of MS2 by this process. Different solar UVA radiation intensities (15, 30 and 45 W m^{-2}) were evaluated using the same initial concentrations of Fe^{3+} and H_2O_2; 1 mg L^{-1} of each during the photo-Fenton process. A slight difference was observed under 15 and 30 W m^{-2} solar exposure, reaching complete inactivation in 25 and 20 min, respectively (Fig. 3a). However, at the higher intensity, the detection limit was reached in less than 2 min. The irradiance plays an important role in the photo-Fenton process since the reaction between Fe^{3+} and H_2O_2 is at least 3-orders

of magnitude faster than in the Fenton reaction [43]. All the iron involved in the catalysts, mainly containing Fe^{3+}, requires UV irradiation to accelerate the reductive generation of Fe^{2+}. The light favors the production of oxidative species since more Fe^{2+} is available to react with the H_2O_2 in the photo-Fenton system. For that reason, when the light absorption diminishes, it results in an inactivation rate decrease.

Also, the variation of the initial concentration of hydrogen peroxide was very significant during the photo-Fenton treatment (Fig. 3b). When a very low concentration of H_2O_2 was employed (0.1 mg L^{-1}) only 1.4-log of virus was inactivated. However, using 1 and 0.5 mg L^{-1} of H_2O_2, total inactivation was achieved in 20 min. The reaction between H_2O_2 and iron by the photo-Fenton reaction leads to the generation of ROS, which cause the denaturing of the proteins that form the capsid of the virus, leading to the loss of activity of the functions involved in virus infection capacity [32]. When the smaller H_2O_2 concentration was used, the photo-Fenton process was inefficient (Fig. 3b). The ROS formed may possibly react with the hydrogen peroxide (which acts as ROS scavenger) and therefore lower quantities of oxidant species are available to attack the coliphage. If a higher H_2O_2 concentration is present, the excess of reactive can react with Fe^{3+} producing more oxidative species, meaning the H_2O_2-ROS competition is negligible. Therefore, the initial concentration of H_2O_2 in the photo-Fenton reaction must be sufficient to assess an adequate efficiency of the process.

The MS2 inactivation rate decreased when the initial concentration of Fe^{3+} decreased during photo-Fenton treatment (Fig. 3c). The detection limit was reached in 20 min when 1 mg L^{-1} of Fe^{3+} was used whilst double the time was necessary when Fe^{3+} concentration was reduced to the half. Only a reduction of 2-log was observed with the lowest iron concentration (0.1 mg L^{-1}). The inactivation is more efficient with the higher concentration of iron, probably due to the formation of virus-iron aggregate being favored and consequently the ROS species are generated close to the virus. However, when iron concentration is limited, the reaction between Fe^{3+} and H_2O_2 (Eq. (2)) is probably preferential to the aggregate formation and therefore ROS can be produced further away from the viruses leading to a lower probability of reaction taking place. In the Fig. 2a and b, Fig. 3b and c it is remarkable that the parameter studied follows an exponential correlation with virus inactivation. This means that in the range studied: i) a small increase in the variable leads to a high response in the MS2 inactivation and ii) the parameters assayed are not under saturated conditions.

The effect of the initial pH (6, 7 and 8) on the photo-Fenton process was evaluated using 1 mg L^{-1} of H_2O_2, 1 mg L^{-1} of Fe^{3+} and 30 W m^{-2} of solar irradiance. Preliminary experiments confirmed that no MS2 inactivation occurred with these values of pH in the CBS solution (data not shown), therefore the inactivation effect only can be attributed to the photo-Fenton treatment. Fig. 4 shows the remarkable differences between inactivation at pH 8 versus pHs 6 and 7. At pH 8, 20 min was needed to reach the detection limit, whilst at the pH 6 and 7 the detection limit was reached in less than 10 min. Other authors observed a marked influence of pH during the inactivation of MS2 virus by the Fenton reaction [28]. Also, H. Lanhua et al., [56] explained that strong pH dependence exists in the treatment of different coliphages like MS2, f2 and Qβ with Fe (IV). Therefore, since some authors have demonstrated that there is strong pH dependence in the virus inactivation, the water matrix used was sterile carbonate buffer solution (CBS) in order to maintain a pH value around 8 throughout the experiments.

3.3. Applicability of the photo-Fenton process for virus inactivation

In order to study virus inactivation in a real water matrix, different assays were carried out in natural water from Lake Geneva (Switzerland). Virus inactivation in natural water and the effect of UVA solar exposure (30 W m^{-2}) were evaluated and any decrease in MS2 coliphage viability was observed (data not shown). In contrast with the results found in the CBS matrix (Fig. 1), the solar exposure/Fe^{3+} process did not show any significant MS2 coliphage inactivation in natural water (data not shown). However, MS2 virus inactivation by the combined effect of solar exposure (30 W m^{-2}) and H_2O_2 (1 mg L^{-1}) or photo-Fenton process was delayed in the real water (Fig. 5). The detection limit was reached 10 min later in solar/H_2O_2 treatment and in the photo-Fenton process it was 2.5 times slower than photo-Fenton carried out in the CBS matrix (Fig. 1).

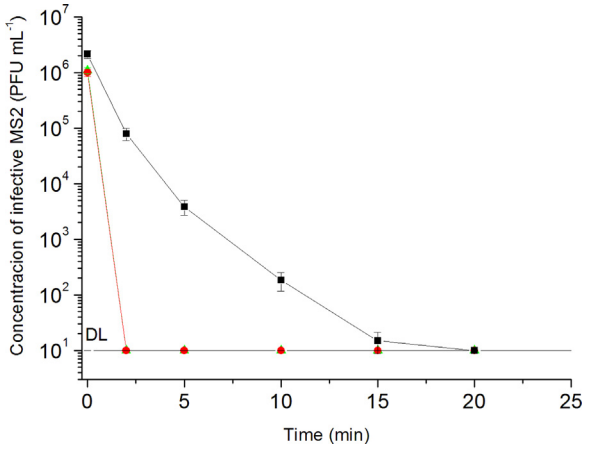

Fig. 4. MS2 Inactivation by photo-Fenton process with 1 mg L^{-1} of H_2O_2, 1 mg L^{-1} of Fe^{3+} and under 30 W m^{-2} of solar intensity at pH 6 (-▲-), pH 7 (-●-) and pH 8 (-■-).

It is well known that natural organic matter (NOM) forms photoactive complexes with iron (which help to keep the iron in solution at neutral pH) that produce ROS via LMCT [4] causing an indirect-exogenous damage to the virus. Rosado-Lausell *et al.* [57] suggested that $^1O_2$, •OH, and the triplet excited state of dissolved organic matter from wastewater or natural water are the main responsible of MS2 virus inactivation. Moreover, during virus inactivation, NOM degradation competes with hydroxyl radical and other ROS produced during photo-inactivation reactions [4,7,8,10,58].

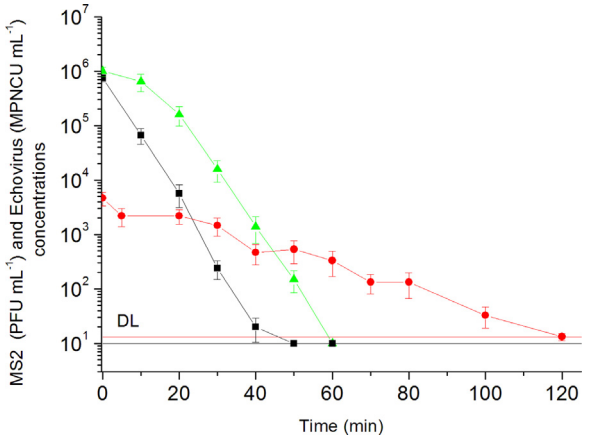

Fig. 5. MS2 Inactivation in natural water from Leman Lake during: UV/H_2O_2 (H_2O_2 = 1 mg L^{-1} and I_{UV} = 30 W m^{-2}) (-▲-) and photo-Fenton (H_2O_2 = 1 mg L^{-1}, $Fe^{2+/3+}$ = 1 mg L^{-1} and I_{UV} = 30 W m^{-2}) at pH 8 (-■-). *Echovirus* inactivation by photo-Fenton (H_2O_2 = 1 mg L^{-1}, $Fe^{2+/3+}$ = 1 mg L^{-1} and I_{UV} = 30 W m^{-2}) at pH 8 in CBS matrix (-●-). Detection limit for MS2 (–) and *Echovirus* (–) concentration measurements.

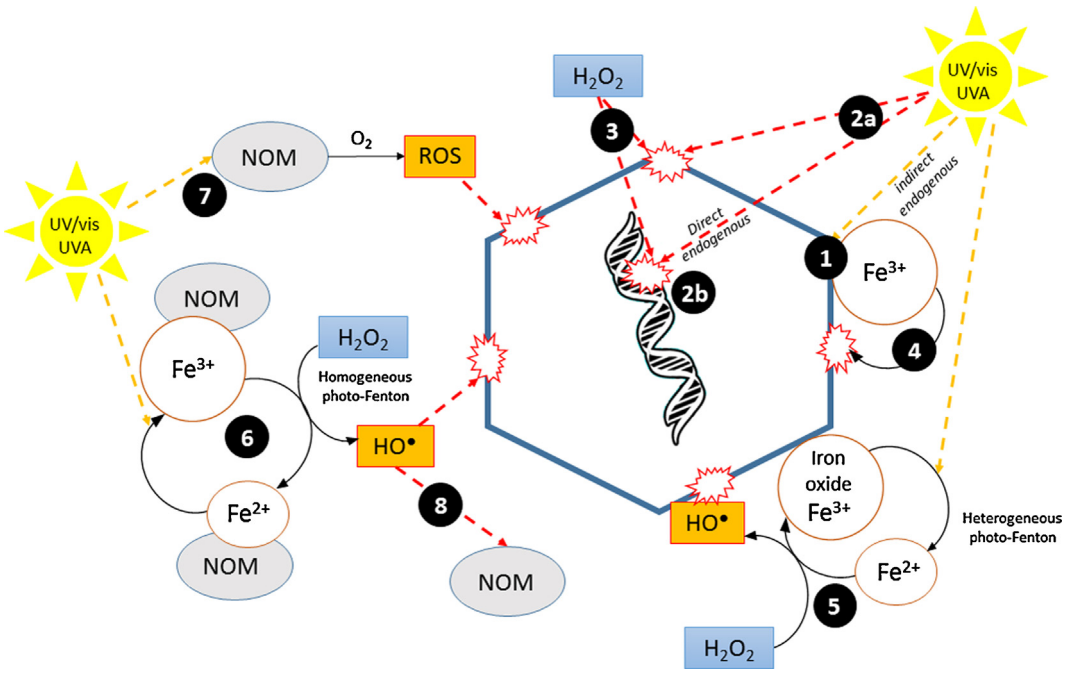

Fig. 6. Mechanistic representation of possible pathways involved in the photo-inactivation of bacteriophage MS2.

Therefore, the clear delay in virus inactivation observed during the photo-Fenton process in the lake water was probably due to the competition between virus inactivation and degradation of NOM present in the natural water for both Fe^{3+}- adsorption and hydroxyl radical or other ROS. The study of the processes involved in the photo-Fenton treatment using the lake water as a matrix could explain the observed effect. In the simulated solar light/Fe^{3+} process, the iron may be predominantly adsorbed onto the NOM instead of the coliphage MS2, which interferes with the formation of the virus-particle aggregate. ROS formed via indirect-exogenous mechanisms are not generated close to the virus and therefore no significant inactivation was achieved. Since the iron present in the natural water was removed by filtration, during the simulated solar light/H_2O_2 process, any decrease in the concentration of infective MS2 can be attributed to photo-Fenton treatment. For this reason, the slower inactivation rate in the solar light/H_2O_2 assay in natural water could be attributed to the competitiveness between the NOM degradation and MS2 virus inactivation for the oxidant agent (H_2O_2). Another cause of loss of virus infectivity was suggested by [30]. They demonstrated that changes in the water matrix such as the presence of humic acids could lead to desorption of the virus from the metal oxide surfaces. This desorption could induce lower virus inactivation, as only the viruses with iron colloids adsorbed are susceptible to being inactivated as mentioned before.

In order to validate the capability of the photo-Fenton process at near neutral pH for human virus inactivation, the photo-inactivation of *Echovirus* was assayed. Up to the date, this has been the first study on human virus inactivation by photo-Fenton. *Echovirus* inactivation by photo-Fenton process with the same iron and hydrogen peroxide concentrations as well as radiation intensity used with MS2 coliphage and using the CBS as matrix were tested (1 mg L^{-1} of Fe^{3+} and 1 mg L^{-1} of H_2O_2 under exposure to 30 W m^{-2} of solar irradiance). As Fig. 5 shows, the detection limit was reached in 120 min of treatment. In this experiment, a high content of organic matter (which could not be removed) was present due to the culture medium. Consequently, apart from the differences related to both viruses, the competition between this organic matter and *Echovirus* for oxidative species would explain why the inactivation rate of *Echovirus* was slower than for MS2 coliphage.

3.4. Mechanistic interpretation

In order to understand the MS2 virus inactivation mechanisms by photo-Fenton, it is important take into account that viral particles attach themselves to receptors on the F-pili of *E. coli* where the genome and portions of the assembly protein are injected into the host cell. Once inside the host cell, MS2 proteins are translated by the host ribosome and virus RNA is transcribed by a complex of MS2 replicase and three host proteins. To be infective, the virus must maintain the following functions: (1) bind to the host cell, (2) inject its genome inside the host cell, and (3) replicate once its genome is inside the host cell [36].

Based on our experimental results and an exhaustive revision of the literature, a mechanistic interpretation of MS2 coliphage inactivation by photo-Fenton reaction is suggested (Fig. 6):

a Physical adsorption of the metal oxides onto the virus surface takes place. The virus-iron hydroxide aggregate is formed mostly by electrostatic interactions since the Fe^{3+} can serve as an electron acceptor and the MS2 coliphage is negatively charged at near neutral pH [11,30].

b a) Solar light causes direct-endogenous damage to the virus components including nucleic acids or proteins which affect the binding of the virus to its host cell, injecting its genome inside the host cell or replicating virus RNA. Nevertheless, b) direct-endogenous damage can be repaired in the host cells [32].

c H_2O_2 causes (in low amounts) oxidative stress in the previously damaged virus as a result of the solar light irradiation [13,58,60].

d The Fe^{3+}-virus complex could act as photo-sensitizer, favoring solar light absorption and resulting in the formation of Fe^{2+} and $^{\bullet}OH$ by a ligand-to-metal-charge-transfer (LMCT), giving rise to indirect-endogenous damage in the virus [11,12,53].

e In the presence of H_2O_2, ROS are generated near the virus as a result of the reduction of adsorbed iron (hydr) oxide [12] by heterogeneous photo-Fenton process with solar light [43].

f In the presence of H_2O_2 and solar light, the photoactive complex formed between Fe^{3+} and NOM leads to homogenous photo-Fenton with the subsequent formation of ROS.

g The exposure of the organic matter present in natural water to solar light, forms a triplet excited state of dissolved organic matter that in the presence of oxygen generates ROS, leading to virus inactivation [57].
h NOM oxidation consumes ROS which compete with virus inactivation [4,7,9,12,57,58].

4. Conclusions

The solar photo-Fenton process at near neutral pH and μM concentrations of H_2O_2 and Fe^{3+} is an effective method for total virus inactivation in natural water. Moreover, the followings aspects make the photo-Fenton process an option of great interest for virus removal: 1) Use of a natural source of energy (sun light), 2) Minimal hydrogen peroxide requirements, i.e., 1 mg L^{-1}, which leads to an significant reduction in the cost of the photo-Fenton process; and 3) Addition of a low cost catalyst such as Fe^{3+}, that is frequently found in natural waters.

Our experimental study suggested that the probable virus adsorption onto iron particles can have a significant effect on MS2 coliphage inactivation efficiency by photo-Fenton treatment at near neutral pH. In this process, ROS generated close to the virus in the presence of Fe^{3+} are more likely to cause damage to the virus than those generated in the bulk of the reaction. Moreover, the source of iron appears to be fundamental to the inactivation since photo-Fenton carried out with Fe^{3+} was more efficient than in the presence of Fe^{2+}. Inactivation rates diminished when photoreactions were carried out in natural water, confirming a competitive effect between virus inactivation and organic matter degradation. Moreover, photo-Fenton treatment at near neutral pH was demonstrated to be effective for human virus inactivation. To our knowledge, this is the first report that presents a mechanistic interpretation of the possible pathways involved in MS2 coliphage inactivation by photo-Fenton process.

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